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Liver fatty acid-binding protein as a biomarker of acute kidney injury after cardiac surgery

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Acute kidney injury (AKI) is a major complication of cardiac bypass surgery. We examined whether levels of liver fatty acid-binding protein (L-FABP) can be an early biomarker for ischemic injury by measuring this protein in the urine of 40 pediatric patients prior to and following cardiopulmonary bypass surgery. AKI was defined as a 50% increase in the serum creatinine from baseline, which was normally not seen until 24–72 h after surgery. Enzyme-linked immunosorbent assay analysis showed increased L-FABP levels (factored for creatinine excretion) of about 94- and 45-fold at 4 and 12 h, respectively, following surgery in the 21 patients who developed AKI with western blot analysis, confirming L-FABP identity. Univariate logistic regression analyses showed that both bypass time and urinary L-FABP were significant independent risk indicators for AKI. After excluding bypass time from the model and using a stepwise multivariate logistic regression analysis, urinary L-FABP levels at 4 h after surgery were an independent risk indicator with the area under the receiver-operating characteristic curve 0.810, sensitivity 0.714, and specificity 0.684 for a 24-fold increase in urinary L-FABP. Our study shows that urinary L-FABP levels represent a sensitive and predictive early biomarker of AKI after cardiac surgery.

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Acute kidney injury (AKI), previously referred to as acute renal failure,[1] is the damage inflicted to the kidney following an insult such as ischemia. The incidence of AKI in children following cardiac surgery is between 5 and 20%.[2,3] Patients who experience AKI following surgery are at a much higher risk of complications and death.[4] Even small degrees of renal dysfunction are associated with increased mortality.[5,6] Pediatric patients comprise an important population for study, since they usually do not have significant comorbidities such as hypertension, atherosclerosis, or diabetes, which affect kidney function in adults. Furthermore, pediatric patients with congenital heart disease undergoing elective surgery are more amenable to study putative urinary biomarkers of AKI, since the time at which renal ischemia occurs after cardiopulmonary bypass (CPB) surgery is well-defined, and these subjects can be studied prospectively for the development of AKI.[7]

In clinical practice, the diagnosis of acute renal failure is usually made by observing an increase in serial measurements of serum creatinine. Unfortunately, serum creatinine is not a sensitive biomarker and occurs only after the disease has progressed. The pathophysiologic processes leading to and following kidney injury activate inflammation, cell death, tubular regeneration, and other responses. One measurable result of these processes is a change in the abundance of certain proteins in the urine. Previous studies using animal models as well as human studies have contributed to our knowledge about cytokines, brush border enzymes, plasma proteins, and other injury-inducible molecules that appear in the urine. A number of potential biomarkers of AKI post cardiac surgery have been proposed including plasma neutrophil gelatinase-associated lipocalin (NGAL) and cystatin C, and a urine panel consisting of NGAL, interleukin-18, and kidney injury molecule-1.[8] Proposed mechanisms for the presence of these low molecular weight proteins in urine include (a) increased systemic inflammation with increased filtration of these proteins in the urine, (b) reduced reabsorption by the damaged proximal tubule, and (c) increased secretion from injured kidney cells into the luminal space.

In recent studies, we have examined the role of human liver fatty acid-binding protein (h-L-FABP) in AKI. L-FABP is a 14-kDa protein normally expressed in human kidney, and, more specifically, in the proximal convoluted and straight tubules.[9,10] In a model of cisplatin-induced AKI, we previously demonstrated increased shedding of urinary L-FABP within the first 24 h, whereas a rise in serum creatinine was not detectable until after 72 h of cisplatin treatment.[11] In this study, we examined the potential of urinary L-FABP as a biomarker of AKI in a human model, namely pediatric patients undergoing cardiac surgery.

RESULTS

Changes in renal function

From 40 patients studied, 21 (52%) developed AKI within a 3-day period. Of these, serum creatinine rose 24–48 h after CPB in 10 subjects, but in the other 11 patients the increase occurred 48–72 h after the procedure. Figure 1 compares the changes in serum creatinine in patients who did not develop AKI with changes with those who developed AKI. As shown in Figure 1, in 19 patients who did not develop AKI, serum creatinine did not change significantly during the first 5 days after cardiac surgery going from 0.52 ± 0.03 at 0 h to 0.41 ± 0.02 mg per 100 ml at day 5 after surgery. In contrast, in subjects who developed AKI, serum creatinine increased from 0.40 ± 0.03 mg per 100 ml before surgery (0 h) to $0.77 \pm$ mg per 100 ml at 48 h post cardiac surgery ($P < 0.01$ when compared to serum creatinine in patients who did not develop AKI). The rise in serum creatinine was sustained for 5 days, suggesting the presence of a sustained form of acute tubular injury such as acute tubular necrosis. On the basis of the primary outcome, we classified children into those with and without AKI. Children who developed AKI tended to be younger and had significantly longer CPB times and length of hospital stay when compared with those who did not develop AKI (Table 1).

Detection of urinary h-L-FABP after cardiac surgery

We performed analysis of urinary h-L-FABP in 40 children who underwent cardiac surgery for correction of congenital malformations. AKI, defined as more than 50% rise in serum creatinine from baseline values, occurred in 21 subjects, but the diagnosis was delayed by 24–72 h after surgery. As shown in Figure 2, in 19 patients who did not develop AKI, a significant increase in urinary h-L-FABP was noted from 36 ± 18 ng mg^{-1} before surgery to 360 ± 104 ng mg^{-1} at 4 h post surgery, and 208 ± 63 ng mg^{-1} Cr at 12 h post surgery. In contrast, subjects who subsequently developed AKI had a much more dramatic increase in urinary h-L-FABP from baseline levels before surgery of 20 ± 4 to 1885 ± 500 ng mg^{-1} Cr at 4 h and 904 ± 320 ng mg^{-1} Cr at 12 h post cardiac surgery.

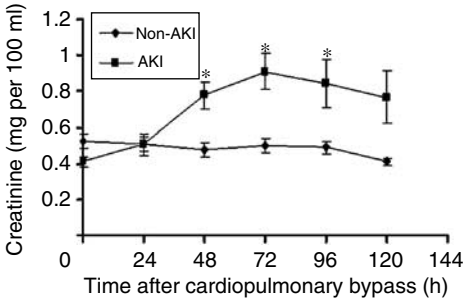

Figure 1 | Changes in serum creatinine (mean ± s.e.) at various time points after cardiac surgery in the non-AKI and AKI group. *$P < 0.01$ when serum creatinine is compared at various time points between non-AKI and AKI patients.

Western blot analysis was also performed in urine samples obtained from children who underwent CPB surgery using a monoclonal antibody raised against h-L-FABP. As shown in Figure 3, the presence of a single band corresponding to the expected size (14 kDa) of h-L-FABP was primarily detected at 4 h but also with less intensity at 12 h after cardiac surgery, only in urine samples obtained from children who did develop AKI. Our western blots results parallel the findings of enzyme-linked immunosorbent assay (ELISA) measurements, indicating that in patients who developed AKI, there is a substantial amount of urinary excretion of L-FABP at 4 h after cardiopulmonary bypass, but the amount of L-FABP was considerably lower at 12 h after cardiopulmonary bypass.

Urinary L-FABP levels predict the development of AKI post cardiac surgery

Our series of analyses began with a Mann–Whitney *U*-test to compare the 4-h post surgery from baseline mean difference in L-FABP levels for AKI patients versus those patients without AKI. The test resulted in a significant mean L-FABP difference (*P*-value = 0.0007) between the AKI patients and those patients without AKI, which supported our hypothesis that at 4 h post surgery, L-FABP levels between the two groups are highly significant in their mean differences from baseline values. The predictability quality of L-FABP for AKI was further explored with an additional compilation of statistical methods.

Univariate logistic regression analyses were performed on the following variables to determine their independent ability to serve as risk indicators for AKI: age, gender, CPB time, previous cardiac surgery, and L-FABP. The univariate logistic regression analyses resulted in two significant independent predictors of AKI: CPB time (*P*-value = 0.0043) and L-FABP (*P*-value = 0.0265). The outcomes of the remaining variables (age, gender, and previous cardiac surgery) were insignificant as independent predictors of AKI. Correlation analyses were performed with Spearman's correlation coefficients to determine the correlation between L-FABP and the following clinical outcomes: percentage of change in serum creatinine (δ-creatinine) and length of hospital stay after surgery. This analysis was performed using all patients, both AKI patients and those patients without AKI, and included all time points (baseline, 4 h post surgery, and 12 h post surgery) for the variables of interest. Both correlation coefficients were statistically significant: δ-creatinine ($r = 0.2617$, *P*-value = 0.0039) and length of hospital stay after surgery

Table 1 | Patients's characteristics and clinical outcomes

	Without acute kidney injury (*n*=19)	Acute kidney injury (*n*=21)	*P*
Demographics			
Age (years)	4.3 (1.3)	2.7 (0.8)	0.233
Boys	12	9	
Girls	7	12	
White ethnic origin	12	21	
African American	7	0	
Clinical outcomes			
Previous heart surgery	5	8	
Cardiopulmonary bypass time (min)	82 (9.7)	145 (12.3)	0.00025
Change in serum creatinine (%)	14.7 (3.4)	190 (39)	0.00008
Length of hospital stay	6.1 (1.4)	20.1 (3.4)	0.005

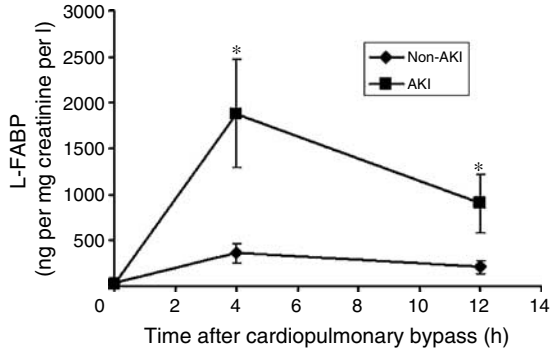

Figure 2 | Changes in urinary L-FABP concentrations at various time points after CPB surgery in non-AKI and AKI patients. Error bars are ± s.e. *$P<0.01$ when urinary L-FABP levels at 4 and 12 h post cardiac surgery are compared between non-AKI and AKI patients.

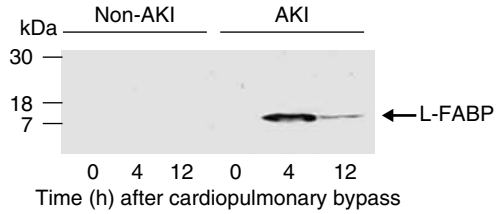

Figure 3 | Representative western blot of urine samples obtained at various time points after CPB from a patient who did not develop AKI and a patient who subsequently developed AKI. Blots were probed with a monoclonal antibody to h-L-FABP.

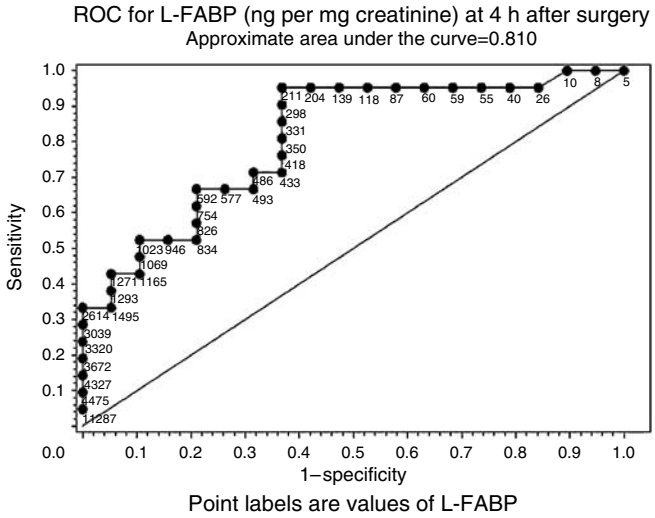

Figure 4 | ROC curve analysis for urinary L-FABP at 4 h post cardiac surgery.

($r=0.32093$, P-value $=0.0004$). Two identical correlation analyses were also performed on the subset of patients at 4 h and 12 h post cardiac surgery. At 4 h post cardiac surgery, the correlation analysis results were as follows: δ-creatinine ($r=0.46544$, P-value $=0.0025$) and length of hospital stay after surgery ($r=0.57825$, P-value <0.0001). At 12 h post cardiac surgery, the correlation analysis results were as follows: δ-creatinine ($r=0.47945$, P-value $=0.0017$) and length of hospital stay after surgery ($r=0.53568$, P-value $=0.0004$). The correlation between L-FABP and both variables (δ-creatinine and length of hospital stay after surgery) were statistically significant when taking all time points into account as well as at both 4 h and 12 h post cardiac surgery. Figure 4 displays the receiver-operating characteristic (ROC) curve for L-FABP at 4 h post cardiac surgery. This figure displays each underlying L-FABP cutpoint value encompassing the ROC curve along with the resulting ROC curve. The area under the L-FABP ROC curve at 4 h post surgery was 0.810. Derived sensitivities and specificities for a selection of specified L-FABP cutpoint concentration levels (211, 350, 486, 592, and 1023) are listed in Table 2. A cutoff value of 486 ng mg^{-1} Cr yields both good sensitivity (0.7142) and specificity (0.6842) levels of AKI at 4 h post cardiac surgery.

A stepwise logistic regression selection procedure was used to determine the most parsimonious model given a set of potential variables for predicting AKI. This analysis was performed on the 4-h post cardiac surgery data, and potential variables for this model included age, gender, CPB time, previous cardiac surgery, and L-FABP. The resulting selected model revealed CPB time (P-value $=0.0043$), as the most powerful independent predictor of AKI in our cohort. When a secondary stepwise logistic regression selection procedure

Table 2 | L-FABP and NGAL test characteristics at different cutoff values

	Sensitivity	Specificity
Cutpoints for L-FABP (ng mg^{-1} Cr)		
211	0.9520	0.6316
350	0.8095	0.6316
486	0.7142	0.6842
592	0.6666	0.7895
1023	0.5238	0.8947
Cutpoints for NGAL (ng mg^{-1} Cr)		
85	1.0000	0.8947
96	1.0000	0.9474
100	1.0000	1.0000
122	0.9524	1.0000
146	0.9048	1.0000

L-FABP, liver fatty acid-binding protein; NGAL, neutrophil gelatinase-associated lipocalin.

was performed including the previous variables while excluding CPB time, this resulted in a most parsimonious model including only the single variable L-FABP (P-value = 0.0265) as a predictor for AKI.

Changes in microalbuminuria

Microalbuminuria was slightly increased in patients who did not develop AKI from levels before surgery of 22.1 ± 5.7 to 47.8 ± 10.1 μg mg^{-1} Cr at 4 h and 72.6 ± 15.9 μg mg^{-1} Cr at 12 h post surgery. In contrast, we detected a significant effect with respect to microalbuminuria in the AKI group. Microalbuminuria increased from levels before surgery of 15.2 ± 4.5 to 328.3 ± 68.3 μg mg^{-1} Cr at 4 h and 586.4 ± 120.3 μg mg^{-1} Cr at 12 h, in those patients who developed AKI. These results are shown in Figure 5.

Changes in urinary NGAL levels

Urinary NGAL, a validated biomarker for AKI,[12] was also measured in this cohort of patients. As shown in Figure 6, urinary NGAL levels were slightly increased in patients who did not develop AKI from levels before surgery of 3.7 ± 0.9 to 32.8 ± 6.1 ng mg^{-1} Cr at 4 h and 21.7 ± 3.8 ng mg^{-1} Cr at 12 h post surgery. In contrast, we detected a significant effect with respect to urinary NGAL levels in the AKI group. Urinary NGAL increased from levels before surgery of 8.0 ± 1.7 to 491.0 ± 82.3 ng mg^{-1} Cr at 4 h and 330 ± 39.8 ng mg^{-1} Cr at 12 h, in those patients who developed AKI. We utilized these measurements to determine the sensitivity and specificity for NGAL at different cutoff values for this identical cohort of patients by constructing a conventional ROC curve. The corresponding area under the curve was calculated for the ROC curve, which yielded a quantifier for the quality of NGAL as a biomarker for AKI. Possible area under the curve values can range from 0 to 1.0, where a value of 1.0 signifies a perfect biomarker and a value of 0.5 is no better than one would expect under random chance. Figure 7 displays the ROC curve for NGAL at 4 h post cardiac surgery. This figure displays each underlying NGAL cutpoint value encompassing

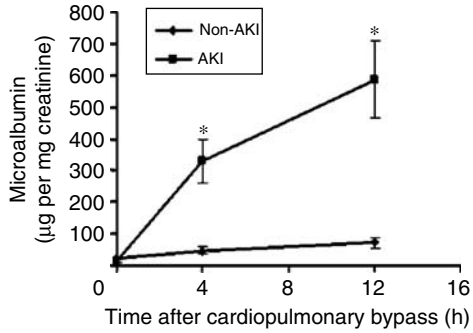

Figure 5 | Changes in microalbuminuria at various time points after CPB surgery in non-AKI and AKI patients. Error bars are ± s.e. *$P < 0.01$ when urinary levels of microalbumin are compared between non-AKI and AKI patients.

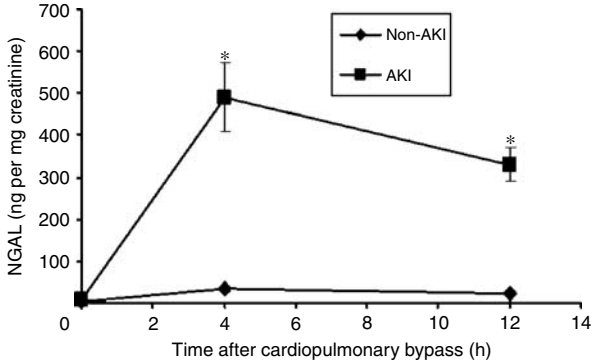

Figure 6 | Changes in urinary NGAL concentrations (mean ± s.e.) at various time points after cardiac surgery in the non-AKI and AKI patients. *$P < 0.01$ when urinary NGAL levels are compared between non-AKI and AKI patients.

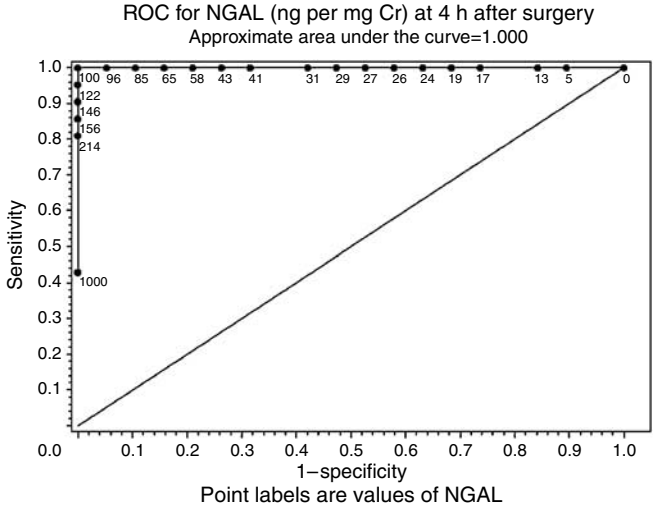

Figure 7 | ROC curve analysis for urinary NGAL at 4 h post cardiac surgery.

the ROC curve along with the resulting ROC curve. The area under the NGAL ROC curve at 4 h post surgery was 1.000, which coincides with the superb, previously published NGAL

results.[12] Derived sensitivities and specificities for a selection of specified NGAL cutpoint concentration levels are listed in Table 2; however, a cutoff value of 100 ng mg^{-1} Cr yielded both perfect sensitivity (1.000) and specificity (1.000) levels of AKI.

Changes in serum L-FABP levels

Because we did not have a large amount of serum samples from our original group of 40 patients, serum L-FABP levels were measured in a total of 16 patients: 8 with AKI and 8 without AKI. Figure 8 presents the data in serum L-FABP levels measured at 0, 4, and 12 h after cardiac surgery. In three out of those eight patients who developed AKI post cardiac surgery, there was significant liver injury determined by an elevation in serum ALT, with levels that varied between 500 and 3000 IU in those three patients. Figure 8 shows that in patients who developed AKI post cardiac surgery, there was a significant increase in serum L-FABP levels at 12 h post cardiac surgery (410.5 ± 120.9 ng ml^{-1}), when compared to serum L-FABP levels measured in patients who did not develop AKI (28.5 ± 8 ng ml^{-1}). In contrast, urinary L-FABP levels measured in this same subset of patients who developed AKI was increased within the first 4 h post surgery from baseline levels of 9.15 ± 1.8 to 791 ± 349 ng mg^{-1} Cr at 4 h post surgery, while urinary levels did not rise in patients who did not develop AKI. These findings suggest that increased urinary L-FABP levels at 4 h post cardiac surgery in AKI patients, rather than just reflecting increased filtration of high serum levels represent an increased in the shedding of proximal tubule L-FABP.

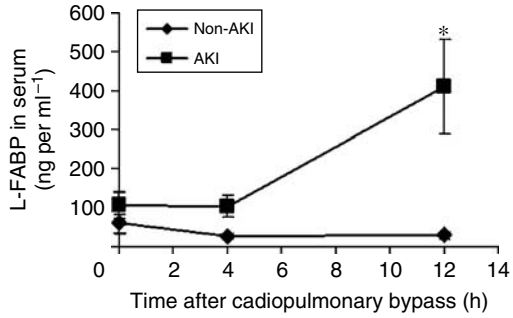

Figure 8 | Changes in serum L-FABP concentrations at various time points after CPB surgery in non-AKI and AKI patients. Error bars are ± s.e. *$P < 0.01$ when serum L-FABP levels are compared between non-AKI and AKI patients.

DISCUSSION

Our study is the first one to demonstrate that urinary excretion of h-L-FABP in pediatric patients undergoing cardiac surgery is significantly increased within the first 4 h of cardiac surgery, and precedes the rise on serum creatinine not seen until 24–72 h post cardiac surgery, in those patients who developed AKI. To evaluate the clinical significance of urinary L-FABP as a biomarker in renal disease, a two-step sandwich ELISA method using monoclonal antibodies to h-L-FABP protein was established for quantification of h-L-FABP in urine.[13,14] There is only one previous study that examined the role of urinary L-FABP in contrast dye-induced AKI.[15] In that study, the investigators found that urinary L-FABP levels were significantly increased only in those patients who developed AKI post contrast dye. One important difference between that study and our study in cardiac surgery patients is that urinary L-FABP levels are much higher in cardiac surgery patients who developed AKI. The discrepancy in the urinary levels of L-FABP between two distinct types of AKI we believe relates to the presence of ischemic injury in patients undergoing cardiac surgery. As demonstrated in a recent study done in Japan,[16] urinary L-FABP levels correlated well to the level of renal ischemia (peritubular capillary ischemia). Therefore, we believe that the degree of ischemia seen in patients undergoing CPB is probably not seen in patients receiving contrast media. Future studies using this urinary L-FABP assay should allow us to further examine these potential differences, as well as the cutoff levels of urinary L-FABP in various forms of AKI.

Our present results lend support to the concept that urinary L-FABP levels can serve clinically as a predictive biomarker of AKI. Other clinical studies have also shown that increased presence of urinary L-FABP can be an excellent clinical marker to predict and monitor the progression of renal disease.[17–23] Kamijo *et al.*[13] reported that various stresses such as massive proteinuria and ischemia induce free fatty acid overload in the proximal tubule and exacerbate tubulointerstitial damage. They also reported[14] that urinary L-FABP levels were more sensitive than proteinuria in predicting the progression of CKD, thereby indicating that urinary L-FABP is a useful clinical biomarker in the monitoring of CKD. In addition, this group reported that the estimated contribution of serum L-FABP to urinary L-FABP in CKD was only 3 ± 3%, suggesting that serum L-FABP levels do not influence urinary L-FABP levels.

Using h-L-FABP transgenic mice and the model of unilateral ureteral obstruction, Kamijo *et al.*[23] showed that h-L-FABP expressed in the proximal tubules, was upregulated in the unilateral ureteral obstruction model of AKI, and that this increased expression likely suppressed the development of tubulointerstitial damage. On the basis of those observations, these investigators concluded that renal L-FABP is likely to be an effective endogenous antioxidant. In more recent studies using h-L-FABP transgenic mice, we demonstrated that cisplatin treatment induced increased shedding of urinary h-L-FABP within the first 24 h and preceded the rise on serum creatinine not seen until 72 h after CP treatment. L-FABPs play a key role in the transport of fatty acids to organelles such as mitochondria and peroxisomes for oxidation and also involved in the regulation of gene expression and cell differentiation.[24–27] Moreover, L-FABP has high affinity and capacity to bind long-chain fatty acid oxidation products and may be an effective endogenous antioxidant.

In addition to urinary L-FABP, our study also shows that the presence of microalbuminuria and increased urinary

NGAL represent additional sensitive biomarkers of AKI during cardiac surgery. We also find that in very few patients who developed AKI post cardiac surgery, serum L-FABP was elevated at 12 but not at 4 h post cardiac surgery, while in these same patients urinary L-FABP was significantly elevated at 4 h post cardiac surgery.

The cause of the elevation in serum L-FABP at 12 h post cardiac surgery cannot be entirely explained by the presence of liver injury post cardiac surgery. Furthermore, our results suggest that increased urinary L-FABP levels at 4 h post cardiac surgery in AKI patients, rather than just reflecting increased filtration of high serum L-FABP levels, represent an increased in the shedding of proximal tubule L-FABP. Further studies will be needed to determine the mechanisms of increased shedding of proximal tubule L-FABP during cardiac surgery.

Our study has several strengths. First, we prospectively recruited a relatively homogeneous cohort of pediatric subjects in whom the only obvious etiology for AKI would be the result of cardiac surgery. These patients comprise an important population for the study of AKI biomarkers, since they do not exhibit common comorbid variables such as diabetes, hypertension, atherosclerosis, and nephrotoxin use. Second, all subjects started with normal kidney function and low levels of h-L-FABP in the urine. The study design allowed for the precise temporal definition of altered h-L-FABP concentrations following cardiac surgery, and a direct comparison with changes in serum creatinine, the current gold standard for the definition of AKI. Our results indicate that h-L-FABP is a powerful early biomarker of AKI that precedes the increase in serum creatinine by several hours to days. The magnitude of rise supports the notion that h-L-FABP is a highly discriminatory biomarker with a wide dynamic range and cutoff values that allow for risk stratification. Indeed, we found that other variables such as patient demographics and previous cardiac surgery were not predictive of AKI, and could not be used for risk assessment in our cohort. Third, we adjusted for potential changes in urinary biomarker concentration by correcting urinary h-L-FABP concentrations using urinary creatinine. Fourth, our results demonstrate that early urinary h-L-FABP levels were not only highly predictive of AKI, but were also associated with important clinical outcomes such as severity of AKI (δ-creatinine) and length of hospital stay.

Our results show significant increases in urinary L-FABP and NGAL levels in children who develop AKI after cardiac surgery. This suggests a common mechanism of proximal tubule injury. Previous studies have shown that after secretion into the tubular lumen, these two lipocalins are reabsorbed by the proximal tubule via megalin-dependent endocytosis.[28,29] Therefore, it is possible that early kidney injury resulting from CPB could cause disruption of megalin-dependent endocytosis in the renal proximal tubule, resulting in the loss of urinary NGAL and L-FABP. A similar mechanism of proximal tubule cell injury was previously described in the animal model of cisplatin-induced AKI.[30]

This study has limitations. First, it is a single-center pilot study of pediatric subjects with congenital heart defects undergoing elective cardiac surgery. Our results will need to be validated in a larger population, including adults with the usual confounding variables and comorbid conditions that normally accumulate with increasing age. Second, ours was a cohort with relatively pristine kidney function, and it will be important to confirm our findings in documented high-risk settings such as preexisting kidney dysfunction, diabetes mellitus, volume depletion, concomitant nephrotoxic drug use, and the hemodynamically compromised patient. Third, in addition to h-L-FABP, simultaneous examination of several potential urinary biomarkers as predictors of AKI will be crucial. Other highly promising urinary candidates include NGAL, kidney injury molecule-1, and interleukin-18. It is likely that not one single biomarker such as h-L-FABP, but a collection of strategically selected candidates, will provide the panel for early and rapid diagnosis of AKI. Fourth, it will be important to partner with industry, to convert our current research-based assays into standardized commercial platforms and point-of-care kits for validation in multicenter trials, and for ultimate clinical utility.

MATERIALS AND METHODS

Patient population

Urine samples were prospectively obtained from 40 patients who underwent cardiac surgery using CPB at Cincinnati Children's Hospital for the correction or palliation of congenital cardiac defects. Exclusion criteria included preexisting renal insufficiency, diabetes mellitus, and concomitant nephrotoxic drug use. AKI was defined as a 50% increase in serum creatinine from baseline, which typically occurred 48–72 h after surgery. For each patient, three urine samples were obtained that corresponded to time 0 h (presurgery), 4 h post surgery, and 12 h post surgery. The research protocol for collection of these samples was approved by the Cincinnati Children's Medical Institutional Review Board, and blind analysis of these de-identified urine samples was also approved by the Institutional Review Board of the University of Arkansas for Medical Sciences. All samples were stored at -80°C prior to analysis.

ELISA for quantitation of urinary L-FABP

The levels of serum and urinary h-L-FABP were measured using h-L-FABP ELISA kit (CMIC Co. Ltd, Tokyo, Japan).[13,31,32] The h-L-FABP protein standard or 50 µl of urine or serum samples obtained from patients were first treated with a pretreatment solution, and then transferred into a 96-well plate coated with a monoclonal antibody against h-L-FABP. After 1 h incubation, the wells were washed and then the conjugate reagent added as secondary antibody for another hour to allow for binding of the h-L-FABP antigen, the immobilized antibody, and the conjugate antibody. After incubation, the plate was washed and a substrate solution for the immunoperoxidase reaction added for 30 min to develop a color based on the amount of h-L-FABP antigen present in the samples. The reaction was stopped using a stop solution. Urinary h-L-FABP concentration was quantitated by measuring the absorbance of each well at 492 nm. Urinary h-L-FABP level was expressed as the ratio of the urinary h-L-FABP in ng mg^{-1} urinary creatinine to adjust for changes in urinary concentration.

Measurement of microalbumin

Urinary microalbumin was measured by standard immunonephelometry (BN Prospec System; Dade Behring Marburg GMBH, Deerfield, IL, USA).

ELISA for quantitation of urinary NGAL

The urine NGAL ELISA was performed using an established and validated assay as previously described.[12] Briefly, microtiter plates precoated with a mouse monoclonal antibody raised against h-NGAL no. HYB211-05; AntibodyShop, Gentofte, Denmark) were blocked with buffer containing 1% bovine serum albumin, coated with 100 µl of samples (urine) or standards (NGAL concentrations ranging from 1 to 1000 ng ml^{-1}), and incubated with a biotinylated monoclonal antibody against h-NGAL (no. HYB211-01B, AntibodyShop) followed by avidin-conjugated HRP (Dako, Carpinteria, CA, USA). TMB substrate (BD Biosciences, San Jose, CA, USA) was added for color development, which was read after 30 min at 450 nm with a microplate reader (Benchmark Plus, Bio-Rad, Hercules, CA, USA). All measurements were made in triplicate. The inter- and intra-assay coefficient variations were $<5\%$ for batched samples analyzed on the same day, and $<10\%$ for the same sample measured 6 months apart. The laboratory investigators were blinded to the sample sources and clinical outcomes until the end of the study.

Immunoblotting

Equal volumes (20 µl) of urine were boiled for 5 min in denaturing buffer and separated on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then electroblotted to a nitrocellulose membrane. The membrane was blocked for 1 h with 5% nonfat dried milk in TBS-T buffer (20 mM Tris, pH 7.6, 100 mM NaCl, 0.1% Tween 20) at room temperature. The membrane was then incubated overnight at 4°C with a mouse monoclonal h-L-FABP antibody (1:2000 dilution) in TBS-T buffer containing in 5% nonfat dried milk. After washing three times with TBS-T buffer, the membranes were incubated with a horseradish peroxidase-conjugated goat anti-mouse IgG as a second antibody (1:5000 dilution) for 1 h at room temperature. Proteins were visualized by using enzyme-linked enhanced chemiluminescence (Amersham, Arlington Heights, IL, USA).

Statistical analysis

A collective set of statistical methods was used to investigate the predictive quality of h-L-FABP as a biomarker for AKI. A Mann–Whitney *U*-test, a nonparametric alternative to an independent two-sample *t*-test, was used to compare the difference between h-L-FABP mean levels in AKI patients and those patients without AKI. Both univariate logistic regression analyses and a stepwise logistic regression analysis were used as exploratory measures for variable predictability of AKI. Potential independent predictor variables included age, gender, CPB time, previous cardiac surgery, and h-L-FABP levels. Furthermore, correlation analyses were conducted by using Spearman's correlation coefficients at post cardiac surgery time points 4 and 12 h as well as an analysis including all time points (baseline, 4 h post surgery, 12 h post surgery). To measure the sensitivity and specificity for L-FABP and NGAL at different cutoff values, a conventional ROC curve was constructed. The area under the curve was calculated for the ROC curve to establish the quality of L-FABP and NGAL as biomarkers for AKI. Possible area under the curve values can range from 0 to 1.0, where a value of 1.0 signifies a perfect biomarker and a value of 0.5 is no better than one would expect under random chance. Race was not included as a factor in any of our analyses due to a complete separation between those patients with and without AKI. A 0.05 significance level was used to determine statistical significance. SAS version 9.1 was used to compute all statistical analyses and figures.

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